A STUDY OF EFFECTS OF OPTIMUM MOISTURE, SATURATION, AND NITRATE LEVELS ON *RHIZOBIUM MELILOTI*

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"A STUDY OF EFFECTS OF OPTIMUM MOISTURE, SATURATION AND NITRATE LEVELS ON RHIZOBIUM MELILOTI"

A DISSERTATION

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF SOIL SCIENCE

by

R. L. THOMAS, B.Sc.

EDMONTON, ALBERTA

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "A Study of Effects of Optimum Moisture, Saturation, and Nitrate Levels on Rhizobium meliloti" submitted by R. L. Thomas, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The effect on rhizobia of excess moisture was indirectly determined by studying its effect on the growth of inoculated alfalfa in a nitrogen deficient sand medium in the greenhouse and by making plate counts of the bacteria to determine their growth in soil and sand cultures in the laboratory. The greenhouse studies indicated that saturation of an inoculated Breton soil prior to seeding was detrimental to the subsequent growth of alfalfa. When sand cultures of alfalfa were grown with continuous saturation, the yield was severely decreased, but alternate weeks of saturation and optimum moisture did not seem to have much effect on the yields of the crop. Laboratory studies concerning the effect of saturation of sand and soil cultures of rhizobia showed little agreement in the numbers of bacteria in sand cultures and in soil cultures, but, in both there was growth and survival of large numbers of rhizobia under the saturated conditions.

The effect of nitrate level and source, and of energy source, on the growth of rhizobia in sand cultures was also studied by plate count experiments in the laboratory. A level of 100 p.p.m. of nitrogen gave maximum growth of the rhizobia, as determined by plate counts. When $Ca(NO_3)_2$, KNO_3 and $NaNO_3$ were compared as sources of nitrogen in the presence of mannitol as the energy source, the numbers of rhizobia obtained with $NaNO_3$ were much higher than numbers obtained with either $Ca(NO_3)_2$ or KNO_3. The energy sources, mannitol and sucrose, were compared in the presence of $Ca(NO_3)_2$ and $NaNO_3$ as nitrogen sources. Numbers of rhizobia obtained and rates of utilization of the nitrate indicated that there was no appreciable difference between mannitol and sucrose as energy sources for rhizobia.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES AND PLATES

INTRODUCTION

Since the characteristic element of protein is nitrogen, the quantity available sets a limit on the life that can be supported. All living matter initially originates from inorganic compounds in the soil and atmosphere, and the amount of nitrogen compounds in the soil (and fixed in the legume nodules) available to plants is the determining factor in the level of protein produced. Although nitrogenous fertilizers have been used for years it is generally recognized that the larger portion of nitrogen must come from the air through biological fixation. Atmospheric nitrogen can only be made available through some method of fixation into compounds that plants can absorb through their roots.

One of the triumphs of modern chemistry has been the combination of atmospheric nitrogen with other elements, the process of "nitrogen fixation". This man-made technique of nitrogen fixation is only a recent discovery, but biological fixation of nitrogen has been going on for countless thousands of years. It is doubtful if the importance of the various biological nitrogen fixation processes is appreciated by more than specialists in the field. There is no doubt however, that the nitrogen fixation processes are important to every living object on earth.

The fixation of nitrogen by microorganisms can be divided into non-symbiotic fixation by free living organisms and the symbiotic fixation by organisms living in nodules on the roots of leguminous plants.

The value of legumes in a permanent system of agriculture has been known to man since ancient times but it was not until 1886 that the mechanism of symbiosis between legumes and microorganisms was discovered. Since that time a great deal of literature has been published on many aspects of the subject. A tremendous amount of work has gone into the development of types of seed inoculants, the improvement of strains of rhizobia, and into methods of inoculation. However, once the legume seed has been sown, the success or failure of the nodulation of the crop depends on the survival of rhizobia in the soil until the legume has a well enough established root system for nodulation to take place.

This investigation was concerned with the survival and reproduction of rhizobia under conditions that could occur during the period when the rhizobia are free living in the soil. The greenhouse part of the investigation was concerned with the ability of the rhizobia to nodulate under excessive amounts of moisture.

LITERATURE REVIEW

In the period since the discovery of the rhizobia-legume relationship by Hellériegel and Wilfarth in 1886 (as reviewed by Fred et al (17)) and the isolation of rhizobia in pure culture by Beijerinck in 1888 (as reviewed by Fred et al (17)), there has been a large and extensive exploration of the field of symbiotic nitrogen fixation. To achieve a complete understanding of the situation, a study must be made of each symbiont individually as well as the relationship between the rhizobia and the legume plant. There have been many reviews of the field of symbiotic nitrogen fixation. Fred et al (17) have reviewed the literature prior to 1932 and Wilson (48) has reviewed the literature on the biochemistry of symbiotic nitrogen fixation up to 1940. Hedlin and Newton (22) and Spencer and Newton (37) have reviewed the development of legume inoculants.

This review will be divided into two main sections, (1) a review of factors affecting primarily the nodulation of legumes, and (2) factors affecting the rhizobia themselves.

1. Factors affecting the nodulation of legumes.

(a) Legume-rhizobia factors.

The ultimate aim of all work on rhizobia is to obtain improved nodulation and fixation of nitrogen in the roots of legumes, thus a review must start with a consideration of the ability of plants to form nodules. Burrill and Hansen (13) investigated the possibility of nodulation of non-leguminous plants. However, all attempts to form nodules on non-legumes failed. Fred et al (17) after reviewing the subject state

that there is no evidence of infection of non-leguminous plants by rhizobia. The phenomenon of nodulation is so general that the inability to form nodules by some legumes has usually been overlooked. Fred et al (17) report that among the subfamily Caesalpinioideae there are several non-nodulating plants. Moore, (as reviewed by Fred et al (17)), claimed that it was possible for the bacteria to enter the roots and benefit the plant even though no nodules are formed. Leonard and Reed, and Gutscly, (as reviewed by Fred et al (17)), found that non-nodulating legumes may attain a nitrogen content comparable to the nodulating legumes. Allen and Baldwin (4) report that only about 88 percent of the members of the family Leguminosae are capable of bearing nodules. The genetic character of the host plant has a bearing on whether it is susceptible to nodulation. It has been shown that some varieties of the various legumes cannot form nodules with rhizobia. Williams and Lynch (47) report on the inheritance of a non-nodulation character in soybeans. Using two sister strains of soybeans, they were able to show that the factor of non-nodulation is genetically controlled and inheritable. Nutman (35) has given a fairly complete review of the influence of the legume in root nodule symbiosis.

Even if nodulation can occur it may not be of value to the host plant. Allen and Baldwin (4) report that some strains of rhizobia are actually parasitic rather than just ineffective. There is a great variation in effectiveness of different strains of rhizobia. Baldwin and Fred (8) tested eighteen strains of Rhizobium trifolii and found that three of these strains were essentially parasitic. There was a gradual gradation from the poorest to the best strains. Baldwin and Fred (8) and Keeney (27) reported that the poorest strains formed more nodules

well distributed over the root system while the better strains formed few nodules concentrated on the upper roots. Fred et al (17) have reviewed the work of a number of authors who report this type of nodulation with strain variation. Keeney (27) also reports that the fastest growing organisms and the ones producing the most gum are the most efficient in fixing nitrogen.

Lynch and Sears (30) compared the response of strains of Lotus corniculatus bacteria in the field and greenhouse, and later in combination with soil treatments. They found that response in the greenhouse will compare favorably with response in the field. In combination with fertilizers they found that the highest yields and quality were obtained by the use of efficient strains at high fertility levels.

Differences in the effectiveness of strains of pea organisms have been reported by Newton and Wyatt (32). Selected strains were much better than strains native to grey-wooded soil in Alberta. The importance of using only effective strains for the first inoculation has been shown by Burton and Allen (15). If clover plants were first inoculated with ineffective strains, reinoculation at a later date with effective strains did not give much benefit. When plants were inoculated with a mixture of an effective strain and an ineffective strain, the resulting plant growth was intermediate between inoculation with either an effective strain or an ineffective strain. Waters (46) states that in natural soil conditions the host plant will likely exert a large influence on the balance between strains.

(b) Environmental factors affecting nodulation.

Giobel (20) states that climate does not exert any effect on nodulation other than its effect on the host plant. Wherever

legumes can grow satisfactorily it seems that nodulation and nitrogen fixation will take place. There are however a number of conditions that will affect nodulation. According to Fred and Davenport (18) there is a difference in acid resistance between species of rhizobia. The alfalfa organisms are the most susceptible to acid conditions and the lupine bacteria the most resistant. Other species are intermediate. Bryan (12) reports that there is very little difference between strains of the soybean organisms. The limits for inoculation that were obtained were pH 4.6 - 8.0. Albrecht (1) states that the degree of acidity is important in excessively acid soils. At a pH of 5.0 or less the failure of nodulation is due to the acidity of the soil. At a high pH the failure to nodulate may be attributed to a deficiency of calcium.

The moisture content of the soil is important to the symbiosis of rhizobia and legumes. Moore (as reviewed by Giobel (20)) states that a high level of moisture is necessary for maximum nodulation and optimum nitrogen fixation. The suggestion is made that, with a higher moisture level, the bacteria have a better opportunity to come in contact with the roots of the legume plants. Wilson, Gain, Perkins, and Prucha (as reviewed by Thornton (39)) all report that an increase in moisture content of the soil, up to the point of water-logging, will favor nodulation. Giobel (20) reports that aeration is very important for nitrogen fixation. Bond (10) in experiments with soybeans grown in water culture reported conflicting results. In one set of experiments, aeration improved nodulated plants, but subsequent experiments could not reproduce the same effect. In these later experiments aeration caused more nodules and longer roots, but produced no change in dry weight or in nitrogen fixation. Kemper and Amemiya (28) measured the oxygen content of the soil atmosphere after

flood irrigation. The lowering of the oxygen content to less than 13 percent for periods of 2 - 3 days did not affect alfalfa growth. In one series where the irrigation was very heavy, the surface of the soil was sealed against diffusion for at least eight days. During this period the oxygen content dropped to about 6 percent with some readings as low as 3 percent. The growth on these plots was better than on adjacent plots that received normal irrigation.

Virtanen and von Hausen (41, 42) reported that nodules were formed in water cultures, but the nodules are small and the plants did not grow very well. If the water cultures were aerated, the plant growth was much better. However, nodulation was completely inhibited if a stream of gaseous nitrogen was passed through the medium. Bond (11) grew plants at three levels of oxygen tension. When the oxygen supply to the roots was reduced, it became the limiting factor more quickly in nodulated root systems depending on the nodules for nitrogen supply, than in non-nodulating root systems supplied with combined nitrogen.

Of the numerous factors that may influence nodule production, the amount and nature of the combined nitrogen in the soil are probably the most important. Fred et al (17) have reviewed a large number of papers on the effect of nitrogen compounds on nodulation. Ohkawara (as reviewed by Fred et al (17) reported that nodulation in sand cultures is prevented by 0.2 percent of nitrates, but lesser amounts in the range of 0.02 to 0.05 percent stimulated nodulation. In sand cultures of alfalfa and vetch, Fred and Graul (as reviewed by Fred et al (17)), found that 15 mg. of nitrate nitrogen as ammonium nitrate in 100 ml. of watering solution was sufficient to prevent nodulation. In soil, however, 30 mg. of nitrate nitrogen as sodium nitrate in 100 ml. still allowed the form-

ation of a few nodules. Allos and Bartholomew (5, 6) reported that nitrogen fixation decreased as fertilizer nitrogen was increased except for very low fertilizer levels where the stimulation of plant growth caused an increase in nitrogen fixation. They also report that fixation never supplies sufficient nitrogen to give maximum plant growth.

2. Factors affecting the survival and growth of rhizobia.

The survival of rhizobia while living free in the soil is of great importance in the ultimate success of legume inoculation. Fortunately, rhizobia have proven able to survive most conditions that occur in soil. Vandecaveye (40) studied the effects of moisture, temperature, and other climatic factors on the survival of Rhizobium leguminosarum. Duplicate pots of sterile soil were used, one set was placed outside, subject to normal climatic conditions and the second set was maintained at optimum moisture in the greenhouse. The results showed that when cultures were added to the sterile soil in the greenhouse, and outside, there was a rapid increase in numbers during the first few weeks, and then a rapid decrease that was independent of moisture content or temperature. In the pots kept outside, following the initial changes in population, there was a distinct correlation between moisture content and counts. When high precipitation flooded the soil the counts dropped seriously. The drop due to flooding was much greater than the drop caused by high temperature and low moisture content. However, the population of rhizobia was sufficient to cause good nodulation in all pots when plants were grown. Albrecht and McCalla (2) studied the longevity of rhizobia in water cultures. In tap water cultures viable organisms were found after 9 months and in one instance after 42 months.

The effect of low moisture was investigated by Burton (14) in a study of methods of inoculation under adverse moisture conditions. The use of molasses as an adherent for inoculating seed that was to be sown in dry conditions was tried. However no treatment could keep the rhizobia alive for more than 2 or 3 weeks in dry soil. Alicante (3) used sugar, soil and glue in an effort to maintain the viability of rhizobia outside the plant. The use of sugar seemed to give some benefit.

Hofer (24) studied commercial cultures and humus cultures for maintainence of numbers. After one week the counts were generally higher than at the time of inoculation. However, after fifty days the counts were lower than that of the original inoculum. After one year counts usually had dropped to 1 percent of the count at the time of inoculation. Hedlin and Newton (22) and Spencer and Newton (37) studied the growth of rhizobia in humus, soil, and humus soil mixtures, and showed that as the moisture content dropped so did the counts of rhizobia. Hedlin and Newton (22) also found that the air supply to humus cultures is important. In tightly stoppered flasks the rhizobia died out in a few days.

The nutritive requirements of rhizobia have also received a great deal of attention, especially in regard to the effect of nitrogen compounds. Hills (as reviewed by Fred et al (17)) demonstrated that there was an appreciable reduction of nitrate nitrogen content of a medium resulting from the growth of rhizobia. Ammonium, sodium, potassium and calcium nitrate stimulated growth of rhizobia in concentrations up to 25 mg. per 100 gm. of soil. There was an increasingly depressing effect on growth up to concentrations in the range of 100 - 150 mg. per 100 gms. of soil. Above this level the nitrates were actually toxic. Spencer and Newton (37) reported that sodium nitrate alone in cultures appeared to stimulate growth but in combination with sucrose there was a very large drop in numbers.

The work of Hofer and Baldwin (23) showed that Rhizobium meliloti responded more to nitrogen in the culture medium than other species of rhizobia. The alfalfa organisms are more vigorous liquefiers of geletin. Walker et al (44) found that yeast extract stimulated growth of Rhizobium leguminosarum as measured by oxygen consumption, but there was no response to the addition of NH_4Cl, $NaNO_3$, urea, or alanine. Further work by Walker et al (45) showed similar response to sources of nitrogen by Rhizobium trifolii and Rhizobium phaseoli. The effect on Rhizobium meliloti was different. Growth as measured by oxygen consumption was considerably increased when nitrogen as NH_4Cl, $NaNO_3$, urea, or alanine was added to the cultures. Spencer (38) also used the rate of oxygen uptake as a measurement of the activity of rhizobia in the soil independent of the host plant. The activity of the rhizobia was stimulated by the addition of ordinary plant resudes.

Like higher plants, rhizobia require a supply of other ions. Fred et al (17) concluded from a study of the literature that calcium, potassium, and phosphorus salts favor the development of rhizobia. The so-called stimulants, boron and manganese, may be favorable in the proper concentrations. Thornton (39) reports that in the complete absence of boron, the vascular tissue that runs out into the nodule becomes disorganized and the nodules do not fix nitrogen. Spencer and Newton (37) report that the addition of phosphorus, potassium or calcium carbonate seemed to increase numbers of organisms. Hedlin and Newton (22) state that a nutrient mixture containing sucrose, calcium carbonate, di-potassium hydrogen phosphate, sodium chloride, and magnesium sulfate, increased the counts of rhizobia in a sterile base. Norris (34) found that the rhizobia of clover or cowpea do not require calcium but the essential element is magnesium. Hoover and Allison (25) report on a growth factor called coenzyme R that greatly increased the growth of rhizobia. Werkman (as reviewed by Fred

et al (17)) found that vitamin B had no effect on the growth rate of rhizobia. In recent work Newton (33) found that molybdenum was required for the successful nodulation of groundnuts in Java.

Rhizobia living in the soil are in competition with other organisms. It has been shown that some strains of rhizobia will compete against other strains. Harris (21) reported that some strains will not form nodules at all when associated with certain other strains. Marshall (31) in an investigation of the competition between strains of Rhizobium trifolii in peat and broth cultures showed that some strains may completely dominate other strains when they are grown together. Waters (46) states that in peat cultures where there is little opportunity for growth, no one strain will dominate the population.

As early as 1899 it was suggested by Nobbe and Hiltner (as reviewed by Fred et al (17)) that competition between other soil organisms and rhizobia results in harmful effects to the rhizobia. Konishi (as reviewed by Fred et al (17)) reported that certain soil bacteria were harmful to rhizobia in liquid culture but not in soil culture. Hedlin and Newton (22) found that counts of rhizobia in unsterilized bases were lower than in sterile bases and concluded that the depression of number of rhizobia was due to the competition from other organisms present. Anderson (7) carried out an investigation on the effect of common soil organisms on the rhizobia-legume relationship and reported no correlation between the organisms harmful to rhizobia cultures and the organisms harmful to the symbiotic relationships.

The overall effect of all factors on the survival of rhizobia has been the subject of several investigations. Vandecaveye (40) reported that once rhizobia had been introduced into Washington soils, nodulation

was still achieved after an absence of 10 - 15 years of the host plant. Von Hausen and Van Glyswyk (43) reported that local strains of rhizobia were better adapted to survival than strains imported from another area. Reid et al (36) dried 40 strains of rhizobia in sterilized soil and stored them for 12 years. On examination it was found that the viability of all cultures was good with the exception of Rhizobium leguminosarum. When corresponding cultures carried on an agar medium were used for comparison, the only change that had occured in the sterilized soil was in Rhizobium japonicum.

MATERIALS AND METHODS

The work reported in this thesis was divided into a greenhouse study of the nodulation of alfalfa, and a laboratory study of the growth and survival of rhizobia under various conditions. The nodulation study was confined to the effect of water saturation as compared to optimum moisture on the normal function of rhizobia. The laboratory study followed two distinct lines:

1. The effect of saturation and optimum moisture in sand or soil cultures of rhizobia.
2. The effect of level and source of nitrate nitrogen on sand cultures of rhizobia at optimum moisture.

Greenhouse Experiments

1. The first experiment was designed to determine whether or not rhizobia retain their ability to nodulate alfalfa after they have been subjected to a period of saturation. For this experiment virgin Breton loam from the vicinity of the Breton plots in Alberta was used. The soil was taken from a 2 - 8 inch depth, thus it was free from most of the organic matter of the A_0 horizon. The air-dry soil was placed in one gallon glazed pots and inoculated in the top two inches with a humus-soil - carbon black culture of Rhizobium meliloti. Sixteen pots were prepared in this manner and set up in four treatments as follows:

Pots 1 - 4 were brought up to optimum moisture for two weeks, then saturated and maintained in a saturated condition for three weeks, and finally drained, seeded to alfalfa, and maintained at optimum moisture for the remainder of the experiment.

Pots 5 - 8 were brought up to optimum moisture at the same time as pots 1 - 4 but were kept at optimum moisture for the full five weeks before seeding. After seeding they were kept at optimum moisture for the remainder of the experiment.

Pots 9 - 12 were prepared two weeks after the first eight pots. They were kept at optimum moisture for two weeks, then saturated and kept in a saturated condition for one week before being drained, seeded, and kept at optimum moisture for the remainder of the experiment.

Pots 13 - 16 were prepared at the same time as pots 9 - 12 but were kept at optimum moisture for the three weeks before seeding, and after seeding until the end of the experiment.

The preparation of all pots was planned so that all of the treatment prior to seeding would be finished at the same time and all seeding could be carried out on the same day. At the end of the experiment the top growth was harvested, dried, and weighed, and the roots were washed out, examined and weighed.

2. The second greenhouse experiment was designed to test the nodulation by rhizobia under various saturation treatments. This experiment was carried out with washed builder's sand, in one gallon glazed crocks that were sterilized prior to the seeding of alfalfa. The alfalfa was allowed to germinate and when the seedlings were about one inch high they were inoculated with an isotonic solution suspension of Rhizobium meliloti. The day after inoculation the following treatments were applied:

Pots 1 - 4 were allowed free drainage of the sand.

Pots 5 - 8 were kept saturated for the entire experiment.

Pots 9 - 12 were drained once a week but otherwise were kept saturated.

Pots 13 - 16 were saturated during alternate weeks, the first week after inoculation being one of saturation.

Pots 17 - 20 were saturated during alternate weeks, the first week after inoculation being one of normal drainage.

The pots were watered daily with a nutrient solution that was prepared in fifty litre quantities as follows:

$Ca(H_2PO_4)_2 . H_2O$	9.0 gms.
$CaCl_2 . 2H_2O$	13.0 gms.
$MgSO_4 . 7H_2O$	15.0 gms.
$K_2 SO_4$	11.0 gms.
Ferric tartrate 1% solution	15 ml.
Boric acid 0.5 p.p.m. B_o	10 ml.
$MnCl_2 . 4H_2O$ 0.5 p.p.m. Mn	10 ml.
$Na_2 MoO_4 . 2H_2O$ 0.1 p.p.m. Mo	10 ml.
H_2O -	50 litres.

At the end of the experiment the top growth was harvested, dried and weighed, and the roots were washed out, examined for nodulation, then dried and weighed.

Laboratory Experiments

Laboratory experiments were set up to follow the growth and survival of Rhizobium meliloti under various conditions. Two experiments were carried out on the effect of water saturation, and three experiments on the effect of source and level of nitrate nitrogen.

Experiment I

This experiment was set up to determine how well the rhizobia survive under saturated conditions. The incubations were carried out in

200 ml. erlenmeyer flasks containing 100 grams of sand of 1.0 - 2.0 millimeter size and 50 grams of sand of less than 1.0 millimeter in size. This had a moisture holding capacity of 20 ml. per 150 grams of sand mixture. The sand mixture was moistened with 10 ml. of a nutrient solution of the same composition as medium 77 of Fred and Waksman (19). Seventy of these flasks were prepared and sterilized for 2 hours at 15 p.s.i., allowed to cool, and inoculated with 10 ml. of a nutrient solution suspension of Rhizobium meliloti. The flasks were incubated at 25°C for one week and then half of them were saturated with nutrient solution. Immediately after saturation the first pair of flasks was plated out with appropriate dilutions. Every day for the first fourteen days and thereafter until the end of the experiment, a pair of flasks was removed from the incubator and plated out.

The method of plating utilized was the standard method of diluting and inoculating plates with one millilitre aliquots. An agar medium was poured and mixed with the inoculum. Generally three dilutions were plated with the middle dilution the one that was expected to be counted. Four replicate plates of each dilution were poured. The medium contained starch, mannitol, congo red, and salts made up in the following proportions:

Agar	15 gms.
Mannitol	10 gms.
Soluble Starch	10 gms.
$CaCO_3$	3 gms.
K_2HPO_4	.5 gms.
$MgSO_4 . 7H_2O$	.2 gms.
NaCl	.1 gm.
Congo Red indicator	10 ml. of 1:400 aqueous solution.

After pouring, the plates were incubated at 25°C for one week and then counted. In counting, only the dilution that gave a count between 30 and 300 colonies per plate was used. The counts reported in almost all cases are averages of four replicate plates.

Experiment 2

This experiment was designed to determine the effect of water saturation on Rhizobium meliloti in soil cultures. The culture medium consisted of 100 grams of virgin Breton loam from the same sample as in the first greenhouse experiment. The soil was placed in 200 ml. erlenmeyer flasks and moistened with 15 ml. of distilled water. The flasks were stoppered with cotton plugs and sterilized at 15 p.s.i. for two hours. The flasks were allowed to sit one week before inoculation so that any reactions in the soil caused by the sterilizing process could come to equilibrium. The flasks were inoculated with 5 ml. of a water suspension of Rhizobium meliloti and incubated at 25°C. Platings were started two days after inoculation and continued daily until the end of the experiment. After the fifth day of plating, half of the remaining flasks were saturated with sterile distilled water, and the plating was continued in the normal way. The method of plating and the medium used were the same as described in experiment one.

Experiment 3

This experiment was set up to test the effect of different levels of nitrate nitrogen on the growth and survival of Rhizobium meliloti. A total of eighty flasks of sand were set up as described in experiment one. The flasks were divided into four groups of twenty flasks each for the four treatments outlined below. The basic nutrient solution in experiment one was amended with the following added levels of nitrate nitrogen applied as KNO_3.

Treatment 1 No nitrogen added.

Treatment 2 25 p.p.m. of NO_3-N.

Treatment 3 100 p.p.m. of NO_3-N.

Treatment 4 400 p.p.m. of NO_3-N.

The flasks were moistened with 20 ml. of the appropriate nutrient solution, sterilized in the autoclave at 15 p.s.i. for two hours, allowed to cool and inoculated with 1 ml. of a water suspension of Rhizobium meliloti. The inoculated flasks were incubated at 25°C. Four flasks, one from each treatment, were removed daily and plated as in the first experiment.

Experiment 4

This experiment, also designed to test the effect of nitrates, utilized two sources of nitrate and a different energy source. A total of 126 flasks of sand were set up as in the previous experiments. The six treatments used were:

Treatment 1 No nitrogen plus mannitol.

Treatment 2 100 p.p.m. NO_3-N as $Ca(NO_3)_2$ plus mannitol.

Treatment 3 400 p.p.m. NO_3-N as $Ca(NO_3)_2$ plus mannitol.

Treatment 4 700 p.p.m. NO_3-N as $Ca(NO_3)_2$ plus mannitol.

Treatment 5 400 p.p.m. NO_3-N as KNO_3 plus mannitol.

Treatment 6 400 p.p.m. NO_3-N as $Ca(NO_3)_2$ plus sucrose.

The basic nutrient solution was medium 77 of Fred and Waksman (19) with the exception of treatment 6 which substituted sucrose for mannitol as the energy source. Each of the flasks of sand was moistened with 20 ml. of the appropriate nutrient solution, sterilized at 15 p.s.i. for two hours, allowed to cool and inoculated with 1 ml. of a water suspension of Rhizobium meliloti. The flasks were incubated at 25°C. Each day one

flask from each treatment was removed and plated according to the method outlined previously. At the time of plating, the first dilution was filtered and an aliquot removed for nitrate determination to follow the level of the nitrates in the culture medium. The nitrates were determined by the phenoldisulfonic acid method as given by Jackson (26).

Experiment 5

This experiment was designed to check the results given by Spencer and Newton (37) on the effect of combination of $NaNO_3$ and sucrose and to compare the effects of $Ca(NO_3)_2$, KNO_3 and $NaNO_3$ as sources of nitrate nitrogen on Rhizobium meliloti. For this, sixty sand flasks were set up as in the previous experiments. The same basic medium 77 of Fred and Waksman (19) was used with the following treatments:

Treatment 1 400 p.p.m. NO_3-N as $Ca(NO_3)_2$ plus mannitol.

Treatment 2 400 p.p.m. NO_3-N as KNO_3 plus mannitol.

Treatment 3 400 p.p.m. NO_3-N as $NaNO_3$ plus mannitol.

Treatment 4 400 p.p.m. NO_3-N as $NaNO_3$ plus sucrose.

The flasks were moistened with 20 ml. of the appropriate nutrient solution, sterilized and inoculated with 1 ml. of a water suspension of Rhizobium meliloti. They were incubated at 25°C and each day one flask of each treatment was removed for plating and nitrate determination as in experiment 4.

RESULTS AND DISCUSSION

(1) Greenhouse Experiments

The yield data of the first greenhouse experiment, which was concerned with the effect of soil saturation on subsequent nodulation and growth of alfalfa in virgin Breton grey wooded soil, are given in Table I. An indication of the differences in growth can be observed in Figure A of Plate I, which was taken about one month before harvest. During the course of the experiment there was a constant difference in the appearance of the plants in the different treatments. The plants of Treatment I, which were subjected to three weeks of saturation before seeding, germinated slower than the plants in the other three treatments, and seemed to be suffering from quite an acute nitrogen deficiency. After about two months of growth the leaves had spotted brown areas, and the stems were more spindly than the check plants of Treatment 2, in which the soil was maintained at optimum moisture prior to seeding the alfalfa. The plants of Treatment 3, in which the rhizobia were subjected to only one week of saturation, had symptoms similar to those that appeared in Treatment 1, but not nearly as severe. The effect of Treatment 1 on yield is apparent. The weight of roots produced was much lower than was produced under the check treatment. The yield of top growth was reduced slightly from the yields of the check, but not nearly to the same extent as the yield of roots.

When the roots were examined for nodules, a general difference between the two check treatments and the two saturated treatments was observed. In the check pots, the nodules appeared in clumps, generally on the upper roots. In the two treatments that had received saturation treatments before drainage of the pots and seeding, the nodules

TABLE I. The effect of optimum moisture and saturation before seeding on Rhizobium meliloti in Breton loam, as measured by subsequent yields of alfalfa*. (Yield in grams).

TREATMENT BEFORE SEEDING		Replicate Pots 1	2	3	4	Ave. Yield
Optimum moisture	Tops	5.55	5.17	6.62	5.92	5.81
2 weeks - saturation	Roots	2.16	2.92	5.73	4.35	3.79
3 weeks	Total	7.71	8.09	12.35	10.27	9.60
Optimum	Tops	6.00	7.43	6.60	7.38	6.85
moisture 5 weeks	Roots	8.83	9.67	11.57	8.70	9.69
	Total	14.83	17.10	18.17	16.08	16.54
Optimum moisture	Tops	6.68	6.82	6.73	6.77	6.75
2 weeks - saturation	Roots	8.99	8.18	7.25	7.76	8.04
1 week	Total	15.67	15.00	13.98	14.53	14.79
Optimum	Tops	7.82	8.48	6.88	6.09	7.32
moisture 3 weeks	Roots	10.56	11.55	10.90	8.42	10.36
	Total	18.38	20.03	17.78	14.51	17.68

* The soil was inoculated with rhizobia, then saturated or held at optimum moisture until seeded and all pots were maintained at optimum moisture after seeding.

PLATE I. GROWTH OF ALFALFA IN GREENHOUSE EXPERIMENTS

Figure A. Growth in virgin Breton soil following various period of saturation of the inoculated soil prior to seeding the alfalfa.

Figure B. Sand cultures of alfalfa subjected to various moisture treatments during the growth of the alfalfa.

were almost all below 3 inches and generally occurred singly rather than in clumps. The importance of such differences in nodulation might be open to question. Conflicting evidence regarding the significance of the size, shape, weight, and color of nodules should not be taken as an indication of their value. Erdman (16), however, classified nodules into three size groups, and found that the larger ones contained ten times as much nitrogen as the small nodules, and so concluded that the larger nodules were of more value to the host plant.

The results of this experiment indicate that a period of saturation before seeding will affect the development of alfalfa grown later. Since it appeared that the alfalfa was suffering from a lack of nitrogen, it might be suggested that the rhizobia and probably also the plant, had been affected in some way that inhibited the fixation of a normal amount of nitrogen. A growth of green algae was observed on the surface of the two sets of pots that had received the saturation treatments. Whether they had any effect, adverse or otherwise, on the plants or the rhizobia was not ascertained.

The yields from the second greenhouse experiment, which was various saturation treatments on alfalfa grown in sand, are given in Table II. Differences in the amount of growth given by the various treatments can be seen in Figure B of Plate I. In Treatment 2, which was continuous saturation, the effect is quite evidently shown by the greatly reduced yields of both tops and roots when compared to Treatment 1 which was maintained at optimum moisture. The amount of aeration given by weekly drainage of saturated pots as in Treatment 3, helped considerably in improving the yields. The average yield of both tops and roots of Treatment 3 is about double the yields of the continuously saturated pots of Treatment 2. The yields of Treatments 1, 4, and 5, which are respectively, optimum moisture, saturated alternate

TABLE II. Effect of various saturation treatments on the growth of inoculated alfalfa in sand cultures. (yield in grams).

TREATMENT		Replicate Pots 1	2	3	4	Ave. Yield
Normal Drainage	Tops	4.69	5.17	5.16	5.97	5.25
	Roots	7.79	8.75	6.85	7.14	7.63
	Total	12.48	13.92	12.01	13.11	12.88
Continuous Saturation	Tops	2.25	1.95	1.85	0.55	1.65
	Roots	1.40	1.21	1.35	0.73	1.17
	Total	3.65	3.16	3.20	1.28	2.82
Continuous saturation, drained weekly	Tops	3.20	3.73	3.20	3.48	3.40
	Roots	2.84	2.28	1.98	1.58	2.17
	Total	6.04	6.01	5.18	5.06	5.57
Saturated alternate weeks (first week is saturation).	Tops	8.08	8.30	7.50	6.85	7.68
	Roots	6.67	6.62	5.63	5.88	6.20
	Total	14.75	14.92	13.13	12.73	13.88
Saturated alternate weeks (first week drained normally)	Tops	5.09	6.12	7.07	5.00	5.82
	Roots	5.34	4.26	6.34	2.89	4.71
	Total	10.43	10.38	13.41	7.89	10.53

weeks starting with saturation, and saturated alternate weeks starting with optimum moisture, are all much higher than either Treatment 2 or 3 which were continuous saturation and continuous saturation with drainage once a week. It seems that alternate weeks at optimum moisture provides sufficient aeration for normal growth.

When the roots were washed free of sand and examined there were quite noticeable differences in both root growth and nodulation as described below:

Treatment 1. Check, with optimum moisture. Very good root growth. The nodulation was good with a large number of small nodules scattered over the finer roots. The heaviest root growth was towards the bottom of the pot, consequently the greatest proportion of the nodules was at a depth of 3 to 6 inches.

Treatment 2. Continuous saturation. The root growth was very restricted, very few roots exceeded 3 inches in length. Most of the nodules on the roots were on the top portion of the roots, many on the main tap root. Most of the nodules were large, grey and spongy in appearance. They were branched and club-shaped rather than round or oval shaped as in Treatment 1.

Treatment 3. Saturation with drainage once a week. The root development was better than in Treatment 2, most of the roots were about 4 - 5 inches long with a larger proportion of finer roots than in Treatment 2. The nodulation appeared similar to that in Treatment 2, with large, grey, spongy nodules on the tap root although a few more nodules appeared on the finer roots than in Treatment 2.

Treatment 4. Saturated alternate weeks starting with a week of saturation. The root development was not quite as extensive as in

Treatment 1, but it was much heavier than either Treatment 2 or 3. The nodules were well scattered over the root system. On the lateral roots the nodules appeared normal, but there was a number of nodules on the main tap root that were abnormal in appearance, similar to the ones in Treatments 2 and 3.

Treatment 5. Saturated alternate weeks starting with a week of drainage. The root development and nodulation were similar to Treatment 4, except there were not as many abnormal nodules on the main tap root. The majority of the nodules were normal in appearance and were on the lateral roots.

It is obvious from the yield data, the observations of the growth as shown in Figure B of Plate I, and the description of the root growth and nodulation that the effectiveness of the rhizobia has been affected by both continuous and intermittent saturation. Since the yields were slightly better in Treatment 3, which was drained once a week, than in Treatment 2 which was continuously saturated, it seems that even the small amount of aeration the roots obtained during the drainage procedure was beneficial. The beneficial effect of aeration is further borne out by the nearly normal growth of Treatments 4 and 5. It is evident that if the plants are to depend on the symbiotic process for their source of nitrogen, then the roots and nodules must be aerated. That normal development of nodules does not occur under saturated conditions is shown by the descriptions of the nodules under the various treatments.

The comparison of Treatments 4 and 5 indicates that saturation immediately following inoculation may affect the nodulation. In Treatments 1 and 5 with normal drainage immediately after inoculation, there were very few nodules on the main tap root. In Treatments 2, 3, and 4, however, with

saturation immediately after inoculation, there was an abundance of abnormal nodules on the main tap root. The location and growth of nodules on the tap root seems to be associated with saturated conditions immediately after inoculation.

The greater yield in Treatment 4 as compared with Treatment 5 is difficult to explain since it appeared that Treatment 4 had a greater number of abnormal nodules. The effects of immediate saturation followed by later drainage, on the inoculation of leguminous plants should be investigated further before any conclusions can be reached.

(2) Laboratory Experiments

(a) Effects of optimum moisture and saturation on numbers of rhizobia.

The results of the comparison of optimum moisture and saturation on numbers of rhizobia in sand cultures are given in Table 3 and illustrated graphically in Figure 1. The counts of rhizobia dropped from the start of plating in both the optimum moisture and saturated flasks. After the first four days under saturated conditions, the counts of rhizobia remained at almost a constant level until the twentieth day when the counts rose somewhat and became increasingly erratic thereafter. The optimum moisture culture numbers also dropped from the initial count, but were generally higher and more erratic than the saturated culture until the fourteenth day. From the fourteenth day until about the twentieth day, the numbers of rhizobia in the optimum moisture cultures were at approximately the same level as in the saturated flasks. After the twentieth day the counts increased and followed an erratic pattern from then on. This increase in numbers of rhizobia may be considered a second growth curve of the culture, possibly caused by nutritional effects or by changes in the stage in the life cycle of the bacteria.

TABLE III. Plate counts of Rhizobium meliloti in sand cultures containing nutrient solution, under conditions of optimum moisture and saturation. (millions per gram)

Incubation Time in days	Optimum moisture	Saturation	Incubation Time in days	Optimum moisture	Saturation
0*	19.9	19.5	22	6.6	5.2
1	13.8	13.0	24	13.0	5.4
2	18.8	9.2	26	10.3	4.4
3	9.8	7.1	28	14.4	6.7
4	15.7	3.8	30	9.8	6.8
5	8.4	4.4	32	14.3	6.8
6	6.7	4.1	34	11.7	6.4
7	5.1	4.3	36	5.8	3.4
8	5.0	3.2	38	11.6	8.2
9	13.0	3.5	40	6.9	6.7
10	4.2	3.0	42	4.6	6.0
11	7.2	4.4	44	4.4	7.3
12	5.8	4.0	46	6.2	6.1
13	6.8	3.6	49	10.1	6.6
14	3.4	3.7	52	5.4	5.2
16	4.7	4.6	54	8.6	7.3
18	3.9	3.2	56	2.28	5.6
20	4.7	4.4			

* The first plating was carried out immediately after the cultures of the saturated series were saturated.

Figure 1 Plate counts of Rhizobium meliloti in sand cultures under conditions of optimum moisture and saturation.

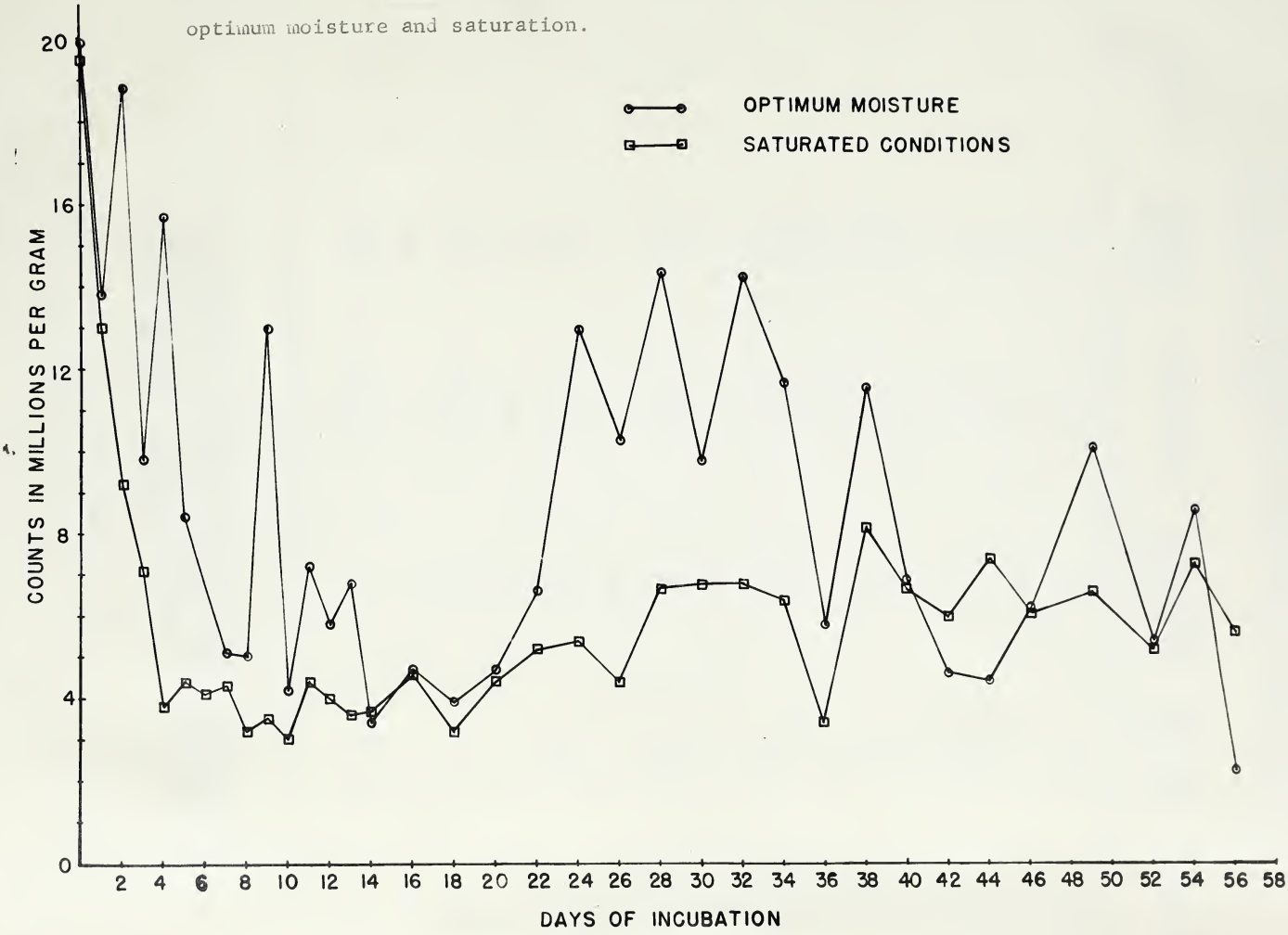
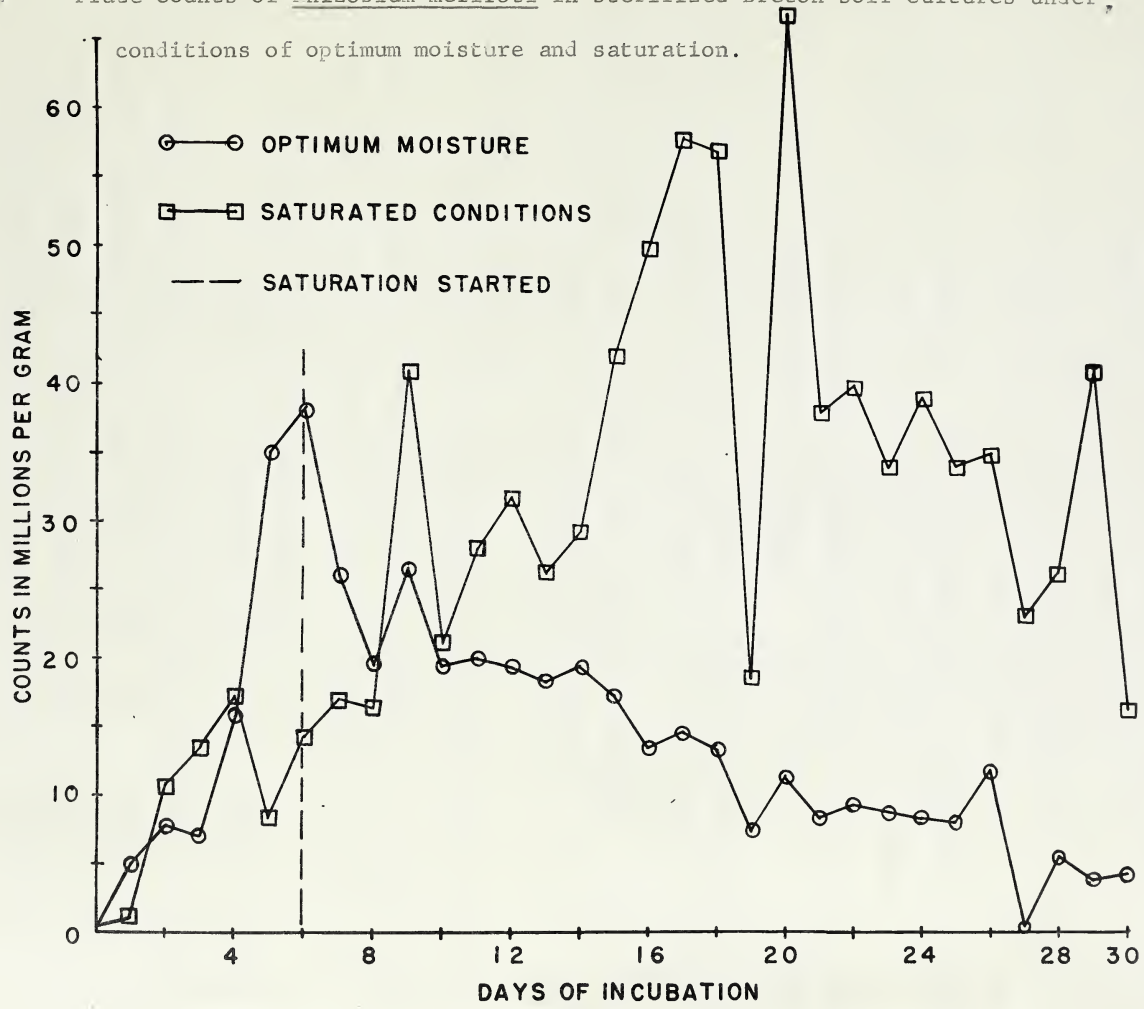


TABLE IV. Plate counts of Rhizobium meliloti at optimum moisture and saturated conditions* in virgin Breton loam cultures (millions per gram).

Incubation time in days	Optimum moisture	Saturation	Incubation time in days	Optimum moisture	Saturation
1	5.0	1.15	16	13.3	50.0
2	7.8	10.7	17	14.5	58.0
3	7.0	13.5	18	13.1	57.0
4	15.8	17.2	19	7.2	18.5
5	35.0	8.2*	20	11.1	67.0
6	38.0	14.2	21	8.4	38.0
7	26.0	17.0	22	9.2	40.0
8	19.4	16.3	23	8.7	34.0
9	26.4	41.0	24	8.2	39.0
10	19.3	21.0	25	5.8	34.0
11	20.0	28.0	26	11.9	35.0
12	19.2	31.6	27	0.46	23.0
13	18.1	26.3	28	5.5	26.2
14	19.3	29.3	29	3.9	41.0
15	17.2	42.0	30	4.2	16.2

* All remaining cultures of the saturated series were saturated just prior to plating on the fifth day of incubation. During the first four days both sets were at optimum moisture.

Figure 4 Plate counts of Rhizobium meliloti in sterilized Breton soil cultures under conditions of optimum moisture and saturation.



The most interesting point shown by this experiment is that the counts of rhizobia remained high even under saturated conditions throughout the entire 56 days of the experiment.

The data of the second experiment utilizing sterilized Breton soil as the culture medium is given in Table 4 and illustrated in Figure 2. Since both sets of flasks were treated alike until one set was saturated on the fifth day, the counts of the first 4 days may be regarded as duplicates. The wide variation between the two counts on the fifth and sixth days was unexpected. This variation must be attributed to biological variation in the growth of the rhizobia in the separate flasks.

After the saturation treatment was started, the general trend of the counts is fairly evident from Figure 2. The counts of the saturated flasks were considerably higher in general than the corresponding flasks at optimum moisture. However, the counts at optimum moisture are not nearly so erratic as the counts under saturated conditions. This condition is exactly the opposite of the condition that existed in the sand cultures of the previous experiment.

Although the pattern of results of the soil cultures does not agree with the previous experiment in sand cultures, it is quite evident that under saturated conditions the rhizobia are able to survive and grow.

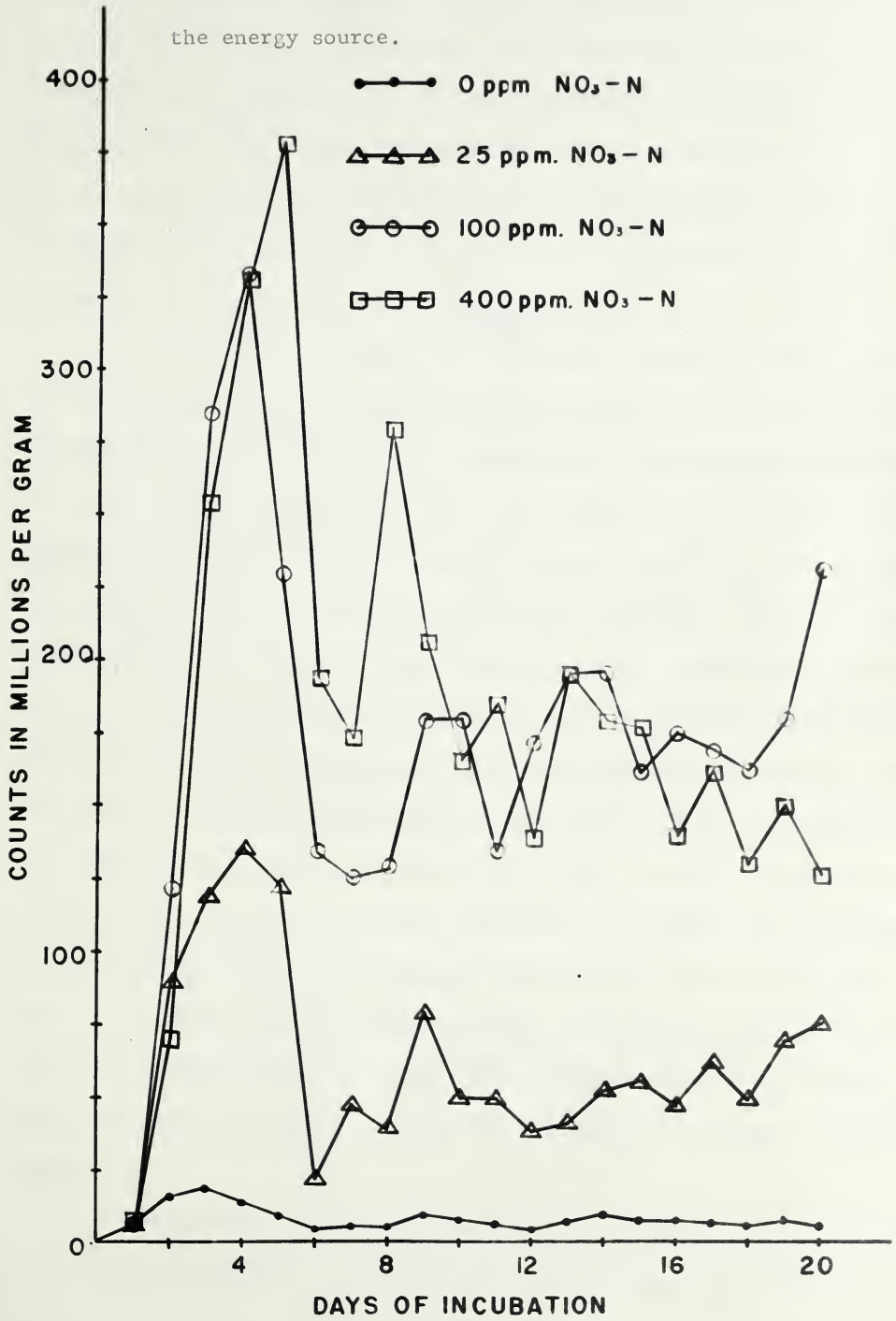
(b) Effects of nitrate level and source.

The results of the first experiment on the effect of nitrate level on numbers of rhizobia are given in Table V and illustrated in Figure 3. In this experiment nitrogen was added as KNO_3. The addition of nitrate nitrogen to the culture medium was definitely stimulating to the rhizobia. In the complete absence of nitrogen the maximum count obtained did not exceed 25 million per gram. The growth curves involving the addition of

TABLE V. Plate counts of Rhizobium meliloti in sand cultures containing a mannitol nutrient solution plus various amounts of nitrate-nitrogen as KNO_3. (millions per gram).

Incubation time in days	0 p.p.m. NO_3-N	25 p.p.m. NO_3-N	100 p.p.m. NO_3-N	400 p.p.m. NO_3-N
1	6.0	5.9	6.5	7.7
2	15.8	89.0	122.0	70.0
3	17.2	117.0	285.0	259.0
4	14.1	135.0	334.0	324.0
5	8.3	123.0	229.0	378.0
6	4.5	21.0	134.0	194.0
7	5.7	47.0	126.0	173.0
8	5.2	39.0	129.0	280.0
9	9.5	77.0	179.0	212.0
10	6.8	49.0	179.0	166.0
11	5.7	49.0	134.0	185.0
12	4.3	37.0	172.0	139.0
13	6.2	40.0	191.0	191.0
14	8.8	52.0	192.0	179.0
15	6.8	55.0	162.0	177.0
16	7.1	47.0	176.0	140.0
17	6.0	62.0	169.0	162.0
18	5.9	48.0	163.0	131.0
19	7.4	68.0	180.0	151.0
20	5.7	76.0	232.0	127.0

Figure 3 Plate counts of Rhizobium meliloti in sand cultures containing various amounts of nitrate nitrogen as KNO_3, with mannitol as the energy source.



nitrate nitrogen are characterized by a rapid rise in numbers, reaching a peak about the fourth or fifth day after inoculation, and then followed by a rapid drop in numbers to a level that seemed fairly constant for the remainder of the experiment. In this experiment there did not seem to be much difference between counts at 100 p.p.m. of nitrogen and at 400 p.p.m. of nitrogen. The indication was that maximum growth might be obtained with 100 p.p.m of nitrate nitrogen. The counts at 25 p.p.m. of nitrogen are definitely below the maximum.

In order to determine the effect of very high nitrate levels on the rhizobia, another experiment was set up. The data obtained in this experiment are given in Table VI. A number of interesting comparisons can be made from this experiment. The first comparison is illustrated in Figure 4, where the counts of rhizobia obtained at 100 p.p.m. are compared with those obtained at 700 p.p.m. of nitrate nitrogen. It can be readily noted that there is very little difference in the total number of rhizobia present under both levels of nitrate nitrogen. This substantiates the results obtained in the previous experiment which indicated that 100 p.p.m. of nitrogen produced maximum counts. The observed rate of disappearance of the added nitrate indicates that at 700 p.p.m. of nitrogen there was still a considerable amount of nitrate remaining at the end of the experiment. This is interesting when compared to the flasks at 100 p.p.m. of nitrogen in which very little nitrate remained after 7 days, yet at both levels of nitrate remarkably similar counts of rhizobia were obtained. Apparently the small amount of added nitrogen was sufficient to produce maximum growth.

When the flasks containing 400 p.p.m. of nitrogen as $Ca(NO_3)_2$ plus mannitol are also considered with the treatments discussed above, it

TABLE VI Plate counts of Rhizobium meliloti in sand cultures containing various nutrient treatments (millions per gram)

Incubation time in days	Treatment										
	0 p.p.m. NO_3-N + mannitol	100 p.p.m. NO_3-N as $Ca(NO_3)_2$ + mannitol		400 p.p.m. NO_3-N as $Ca(NO_3)_2$ + mannitol		700 p.p.m. NO_3-N as $Ca(NO_3)_2$ + mannitol		400 p.p.m. NO_3-N as KNO_3 + mannitol		400 p.p.m. NO_3-N as $Ca(NO_3)_2$ + sucrose	
	Counts	Counts	p.p.m. of N left	Counts	p.p.m. of N left	Counts	p.p.m. of N left	Counts	p.p.m. of N left	Counts	p.p.m. of N left
1	4.1	7.1	28.5	6.5	106.5	4.6	208.5	7.6	103.5	4.2	142.5
2	13.7	48.0	15.0	47.0	108.8	58.0	249.0	282.0	44.2	48.0	150.0
3	22.5	145.0	7.7	109.0	135.8	105.0	277.5	750.0	5.8	71.0	103.5
4	11.6	115.0	2.2	102.0	88.5	74.0	234.0	277.0	4.0	94.0	54.4
5	13.0	83.0	1.4	153.0	31.9	75.0	182.2	254.0	4.3	105.0	31.9
6	22.3	221.0	3.1	194.0	25.9	209.0	221.2	251.0	4.0	178.0	19.9
7	12.9	129.0	1.6	161.0	5.8	129.0	125.2	214.0	3.6	134.0	7.7
8	16.4	128.0	0.8	171.0	7.6	197.0	160.0	196.0	4.6	170.0	28.5
9	12.9	128.0	0.8	170.0	12.6	137.0	107.2	148.0	2.4	154.0	4.8
10	3.3	98.0	0	128.0	8.5	154.0	176.8	150.0	2.4	163.0	6.7
11	5.2	109.0		141.0	6.5	120.0	164.8	118.0	3.5	105.0	11.8
12	8.8	137.0		130.0	6.2	129.0	174.4	138.0	3.2	115.0	7.0
13	9.4	215.0		137.0	13.8	105.0	176.8	127.0	3.4	152.0	9.5
14	7.5	210.0		189.0	7.3	95.0	92.0	216.0	1.0	201.0	4.6
15	3.7	134.0		124.0	4.8	54.0	35.2	98.0	3.2	100.0	4.8
16	4.6	168.0		81.0	7.0	143.0	87.2	100.0	4.2	163.0	7.6
17	4.8	129.0		90.0	2.6	69.0	72.4	118.0	0.6	145.0	4.2
18	2.15	76.0		82.0	3.6	39.0	39.2	111.0	1.4	103.0	4.6
19	3.9	135.0		166.0	11.2	86.0	111.2	83.0	5.4	88.0	7.1
20	3.3	124.0		66.0	4.8	35.0	70.4	78.0	4.9	97.0	6.5
21	5.3	167.0		86.0	6.1	101.0	102.4	108.0	3.8	-	7.7

Figure 4 Plate counts of Rhizobium meliloti and rate of nitrate utilization in sand cultures containing various levels of nitrate nitrogen as $Ca(NO_3)_2$, with mannitol as the energy source.

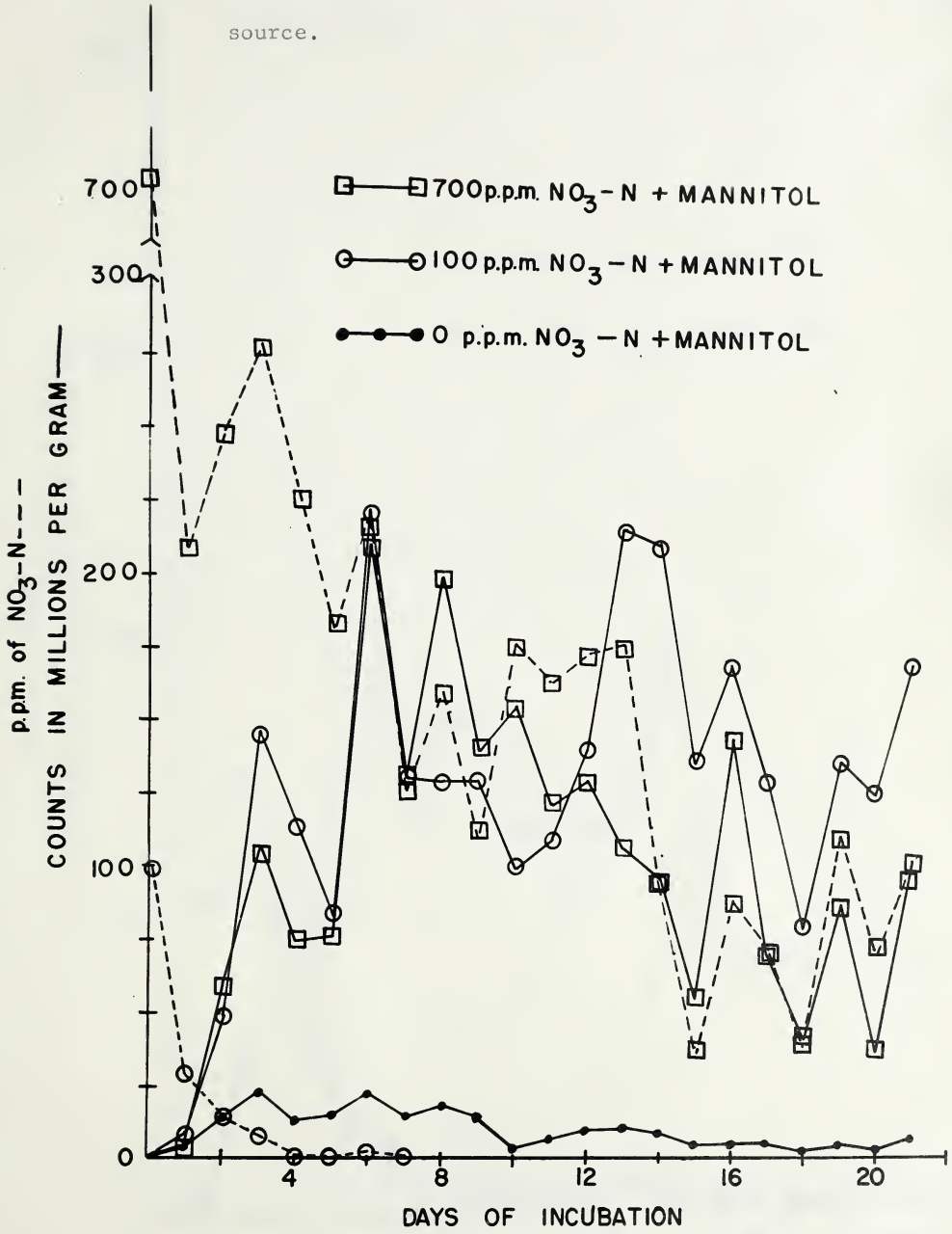


Figure 5 Plate counts of Rhizobium meliloti and rate of nitrate utilization in sand cultures containing 400 p.p.m. of nitrogen as $Ca(NO_3)_2$, with mannitol or sucrose as the energy sources.

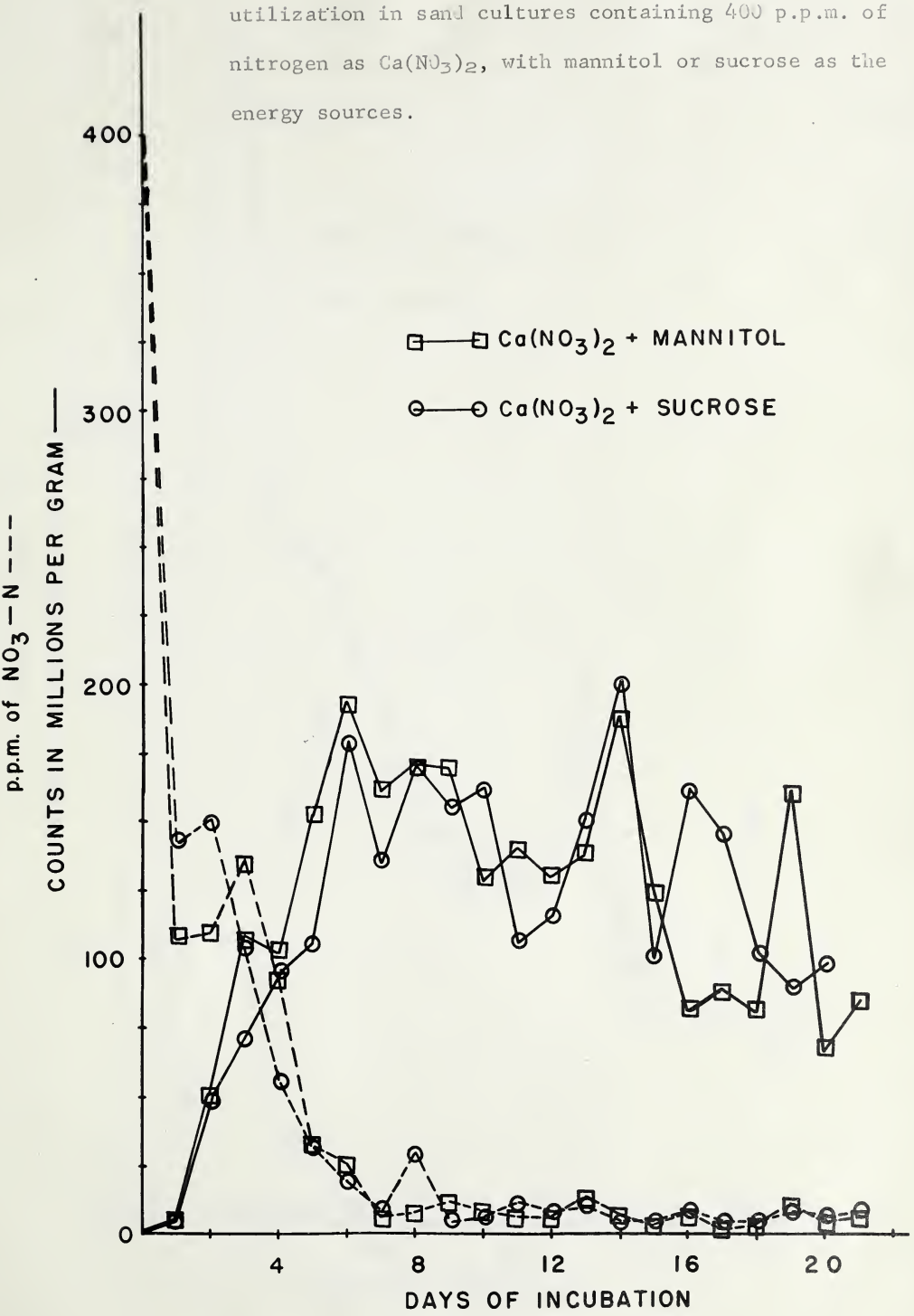
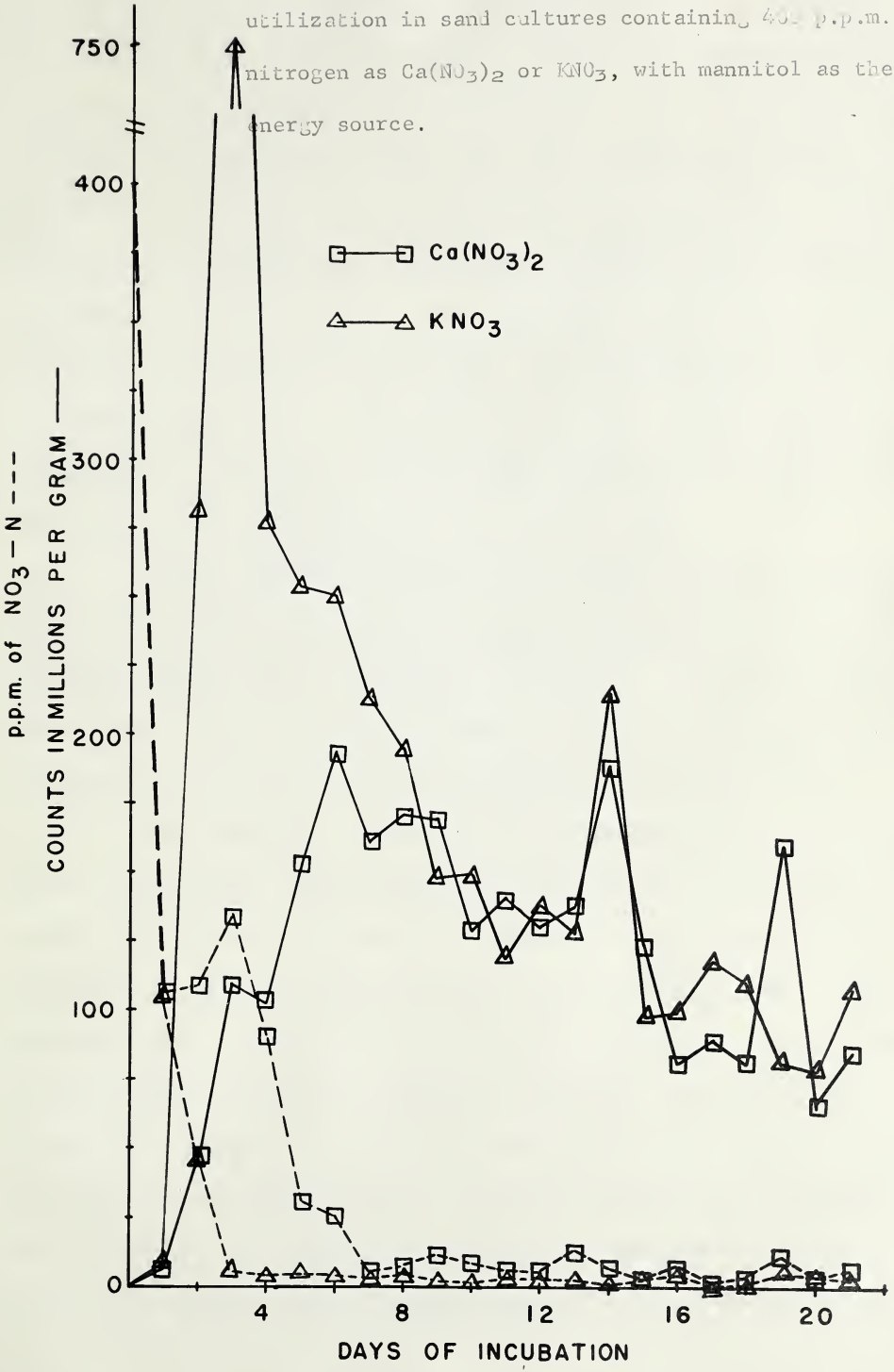


Figure 6 Plate counts of Rhizobium meliloti and rate of nitrate utilization in sand cultures containing 400 p.p.m. of nitrogen as $Ca(NO_3)_2$ or KNO_3, with mannitol as the energy source.



can be observed that this level of nitrogen gave almost the same counts as when 100 p.p.m. or 700 p.p.m. of nitrogen was used. In this case most of the nitrate had disappeared after 7 days and what remained did not disappear during the course of the experiment. It is surprising that in these three levels of nitrogen the counts of rhizobia were very similar, yet in the lowest level all the nitrate had disappeared after 7 days, in the second level there was a small amount remaining until the end of the experiment, and at the highest level there was a considerable amount remaining at the end of the experiment. The disappearance of the nitrate is probably caused by the oxidation of the sugar. Apparently 100 p.p.m. of nitrogen is sufficient to give maximum growth even though all measureable amounts had disappeared after the first 7 days.

Another comparison illustrated in Figure 5, is the effect on the counts of rhizobia of the two energy sources, mannitol and sucrose, when combined with 400 p.p.m. of nitrogen as $Ca(NO_3)_2$. It is easily observed that in both numbers and rate of nitrate utilization there is very little difference in the use of sucrose or mannitol as an energy source.

This experiment also allows one to compare $Ca(NO_3)_2$ and KNO_3 as sources of nitrogen. This comparison at 400 p.p.m. of nitrogen in Figure 6 showed that the KNO_3 stimulated the growth to a considerably greater extent than did the $Ca(NO_3)_2$ during the first week of the experiment. Since the maximum count with KNO_3 was 750 million per gram compared to a maximum of 200 million per gram with $Ca(NO_3)_2$, there is a very significant difference. The rate of nitrate utilization also seems slightly different. With KNO_3 it took only 3 days for the nitrate content of the medium to drop below 10 p.p.m. of nitrogen remaining, while with $Ca(NO_3)_2$ it was 8 days before the nitrogen content dropped below 10 p.p.m. of nitrogen remaining.

The response to KNO_3 indicates that it is more stimulating to rhizobia than $Ca(NO_3)_2$.

The work of Spencer and Newton (37) indicated that a combination of sucrose and $NaNO_3$ was detrimental to the growth of rhizobia. To verify the results of the previous experiment and to check the results of Spencer and Newton (37) an experiment was set up using $Ca(NO_3)_2$, KNO_3, and $NaNO_3$, as nitrogen sources at 400 p.p.m. of nitrogen and with mannitol as an energy source, and $NaNO_3$ at 400 p.p.m. of nitrogen with sucrose as the energy source. The data for these treatments are given in Table VII and various comparisons are made in Figures 7, 8, and 9. Figure 7 compares $Ca(NO_3)_2$, KNO_3, and $NaNO_3$ as sources of nitrogen with mannitol as the energy source. As in the previous experiment KNO_3 stimulated the growth much more than $Ca(NO_3)_2$. However, the response to $NaNO_3$ was much greater than the response to either $Ca(NO_3)_2$ or KNO_3. Since the highest count with $NaNO_3$ is about 800 million per gram and the highest with KNO_3 is only 500 million per gram it seems that $NaNO_3$ is the best source of nitrogen. However, the actual significance of this difference is questionable.

The rates at which all of these sources of nitrate are utilized are compared in Figure 8. There does not seem to be any difference in the rate of utilization.

The effects of the energy sources, mannitol and sucrose, in the presence of $NaNO_3$ are compared in Figure 9. It seems that there was a slightly higher growth with mannitol than with sucrose. However, since after this initial peak, the count with sucrose is slightly higher for the remainder of the experiment, it is hard to state which is the better source of energy when combined with $NaNO_3$. These results are very contradictory to those obtained by Spencer and Newton (37), who report that the combination

TABLE VII Plate counts of Rhizobium meliloti in sand cultures containing nitrate nitrogen from various sources with sucrose and mannitol as the source of energy. (millions per gram).

Incubation time in days	TREATMENT							
	400 p.p.m. NO_3-N as $Ca(NO_3)_2$ + mannitol		400 p.p.m. NO_3-N as KNO_3 + mannitol		400 p.p.m. NO_3-N as $NaNO_3$ + mannitol		400 p.p.m. NO_3-N as $NaNO_3$ + sucrose	
	Counts	p.p.m. of N left	Counts	p.p.m. of N left	Counts	p.p.m. of N left	Counts	p.p.m. of N left
1	1.2	128.0	1.0	144.0	1.0	144.0	1.4	140.8
2	50.0	132.8	48.0	121.6	62.0	156.8	67.0	99.2
3	217.0	24.8	490.0	12.8	880.0	4.6	700.0	4.8
4	259.0	14.8	400.0	2.4	550.0	1.7	630.0	3.2
5	269.0	5.4	284.0	2.9	320.0	2.1	380.0	2.5
6	231.0	4.2	228.0	2.1	239.0	2.7	227.0	2.3
7	420.0	6.6	260.0	2.0	284.0	2.7	410.0	1.9
8	350.0	3.9	216.0	2.7	261.0	1.9	310.0	1.3
9	330.0	3.9	219.0	2.3	270.0	1.7	300.0	1.2
10	340.0	3.6	245.0	1.8	256.0	2.0	320.0	2.1
11	340.0	2.8	350.0	1.6	278.0	2.4	360.0	1.6
12	310.0	2.4	286.0	0.8	272.0	0.8	350.0	0.9
13	290.0	2.5	420.0	0.7	300.0	1.1	340.0	1.2
14	380.0	2.1	510.0	2.8	320.0	1.9	320.0	1.6
15	310.0	4.2	213.0	2.0	280.0	1.2	310.0	1.2

Figure 7 Plate counts of Rhizobium meliloti in sand cultures containing 400 p.p.m. of nitrogen as $Ca(NO_3)_2$, KNO_3 or $NaNO_3$, with mannitol as the energy source.

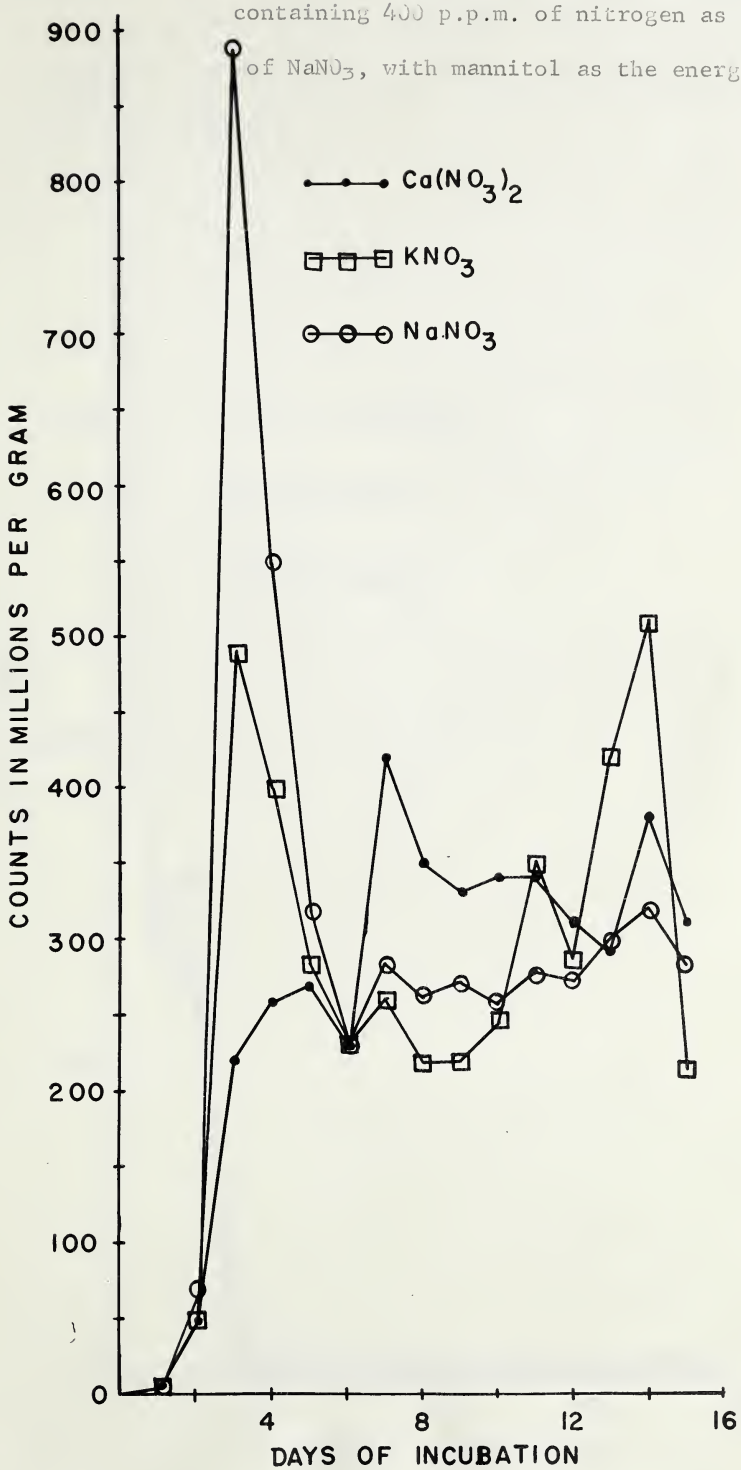


Figure 3 Rates of nitrate utilization by Rhizobium meliloti in sand cultures containing 400 p.p.m. of nitrogen as $Ca(NO_3)_2$, KNO_3, or $NaNO_3$, with mannitol as the energy source.

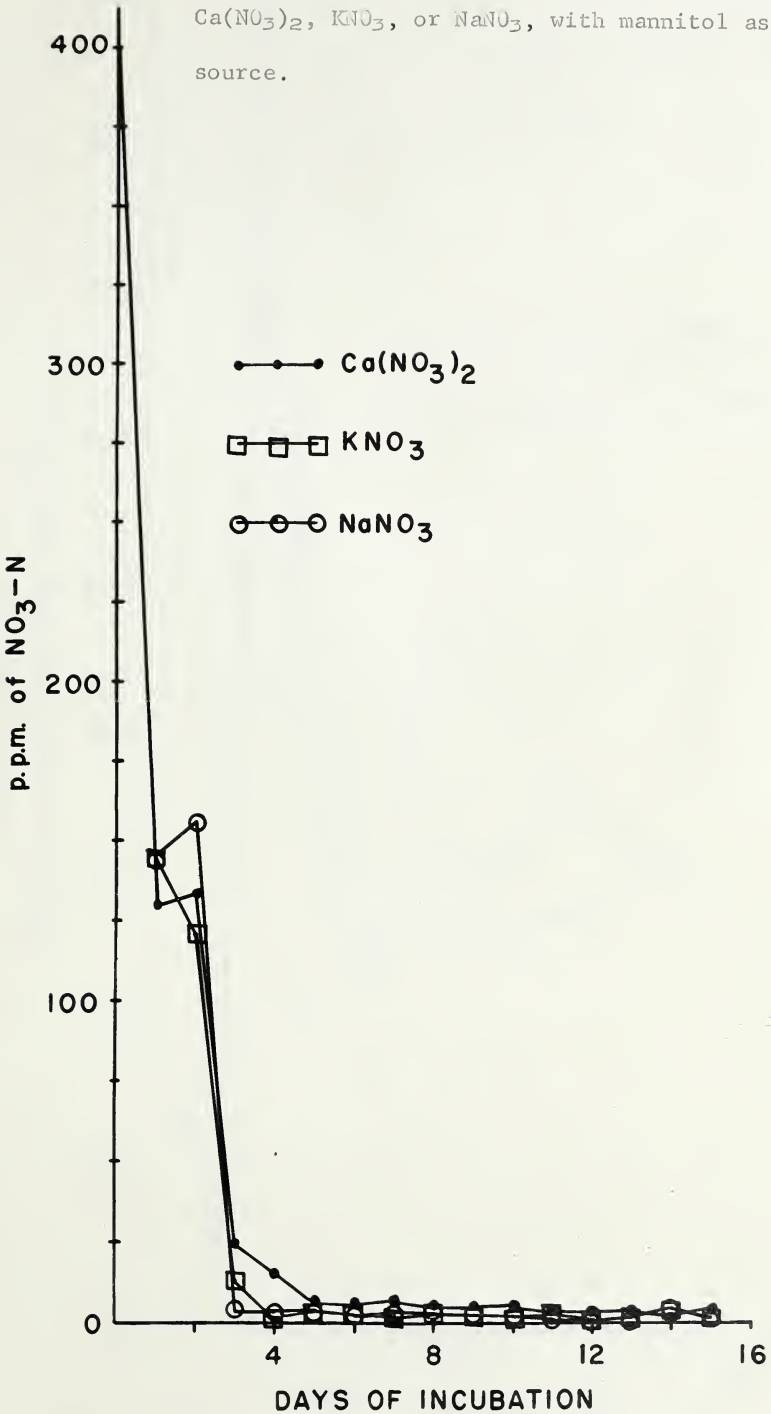
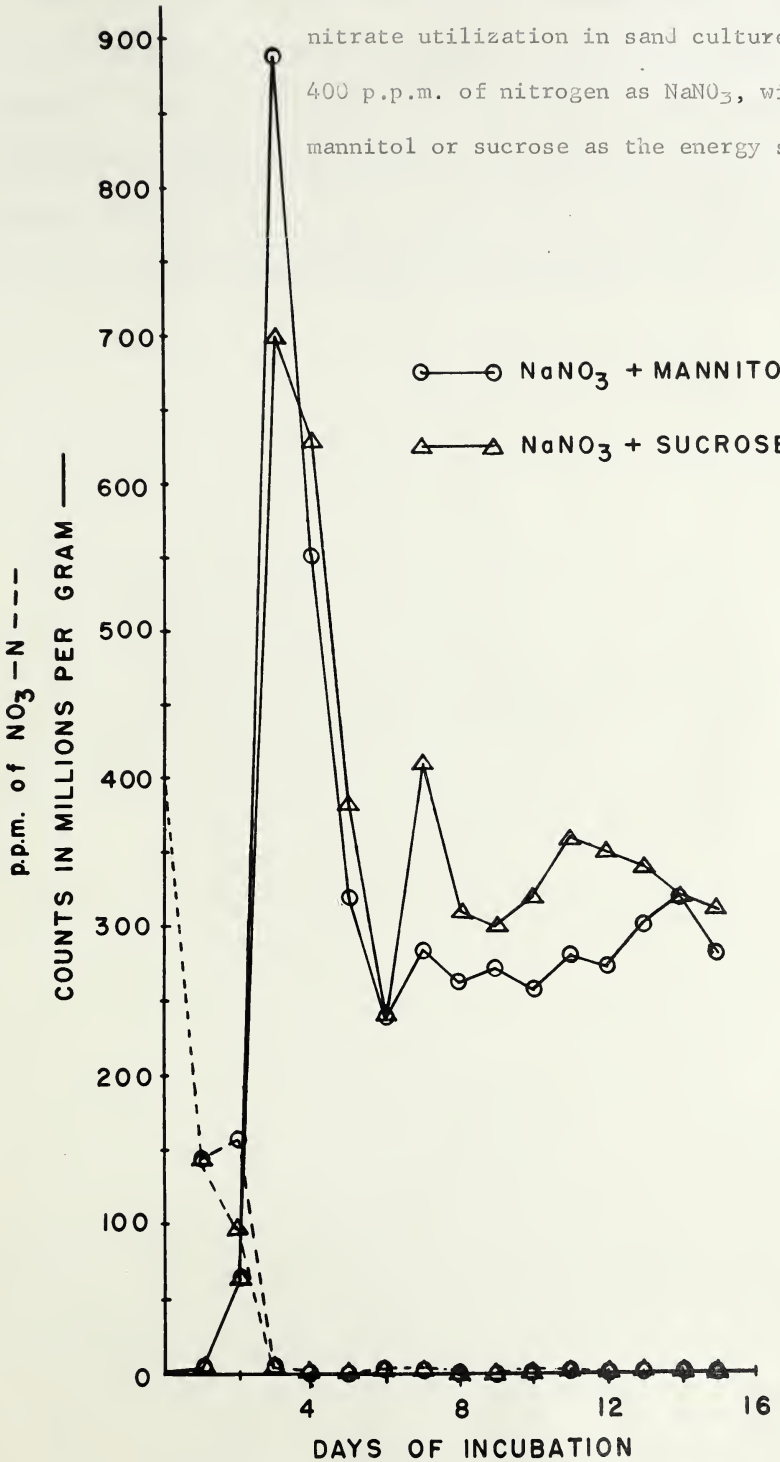


Figure 9 Plate counts of Rhizobium meliloti and rates of nitrate utilization in sand cultures containing 400 p.p.m. of nitrogen as $NaNO_3$, with either mannitol or sucrose as the energy source.



of $NaNO_3$ and sucrose is detrimental to rhizobia and further experimental work to determine the cause of this discrepancy is desirable since Spencer and Newton (37) used a soil-humus base rather than sand. A comparison of the rate of nitrate utilization with the two energy sources indicated that there was very little difference between mannitol and sucrose as energy sources for rhizobia in the presence of $NaNO_3$.

SUMMARY

1. A study was made of the growth of rhizobia and nodulation of alfalfa under conditions of optimum moisture and saturation.
2. In greenhouse studies, the saturation of soil prior to seeding appears to be detrimental to the growth of a leguminous crop.
3. Saturation of sand cultures of alfalfa greatly reduced yields, but alternate weeks of saturation and optimum moisture did not affect yields.
4. In saturated sand cultures the counts of rhizobia were slightly lower than counts of cultures at optimum moisture.
5. In soil cultures the bacterial counts were somewhat higher under the saturated conditions than under the optimum moisture level.
6. A study of the effect of source and level of nitrate nitrogen was made.
7. A level of 100 p.p.m. of nitrogen seems to give maximum numbers of rhizobia since higher levels did not increase the numbers of rhizobia.
8. KNO_3 was a better source of nitrogen for the rhizobia than $Ca(NO_3)_2$.
9. Greater growth in numbers of rhizobia was obtained with $NaNO_3$ than with either KNO_3 or $Ca(NO_3)_2$ as the nitrogen source.
10. Mannitol and sucrose gave similar results as energy sources with $NaNO_3$ or $Ca(NO_3)_2$ as the source of nitrogen.
11. $NaNO_3$ and sucrose were not detrimental to rhizobial reproduction and growth under the conditions of these experiments.

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